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# Elimination of a reproductive barrier facilitates intergeneric hybridization of *Sorghum bicolor* and *Saccharum*

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Abstract:

Growing interest in bioenergy production has increased efforts to breed for greater biomass through intra- and inter-generic hybridization. Both sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum spp.*) are now being bred to enhance the quantity and quality of biomass while maintaining or improving biotic and abiotic stress tolerances. The ability to consistently hybridize these species would facilitate the introgression of complementary traits that increase adaptability, yields, and sustainability of each species. Previous efforts to hybridize these crops have had limited success, but the discovery of a specific trait in sorghum has eliminated at least one prezygotic barrier to fertilization. Techniques to produce a significant amount of seed from crosses between sorghum and sugarcane are described. Using these methods, our programs have grown 1,371 sorghum/saccharum intergeneric hybrid plants. Seed set frequency in the intergeneric crosses was affected by sugarcane pollinators, implying that breeding and selection of sugarcane pollen parents could further enhance successful hybridization. The *Sorghum x Saccharum* hybrids described in this paper are now being used for introgression of traits into both species. Unlike previous attempts to hybridize these two genera, sufficient quantities of seedlings were produced to impose selection criteria with the goal of developing a new intergeneric cultivar with potential to be used for sugar or as a biomass feedstock. The long-term objective is to combine desirable traits of both sorghum and sugarcane.

**INTRODUCTION**

Both sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum sp.*) have been identified as potentially dedicated bioenergy crops. Consequently, there have been increased efforts to develop sorghum and sugarcane germplasm with improved biomass quantity and quality. An ideal bioenergy crop has numerous characteristics which include but are not limited to high yield and quality input requirements that are as low as possible and stress tolerance (Perlack et al., 2005). Biomass feedstocks have been explored in the past as a source of renewable energy, and today there are increasing numbers of studies assessing their strengths and weaknesses (Lipinsky, 1978; Clark et al., 1981; Goldemberg, 2007; Burner et al., 2009).

Crop improvement through breeding relies on genetic variation within the species. When this variation does not exist or is limited, breeders turn toward wide hybridization or transgenic approaches to exploit genes from other sources. Transgenic approaches are effective for traits influenced by only a few genes and typically target a very specific trait. In addition, regulatory approval is cost prohibitive and public perception is sometimes a problem. For traits that are quantitatively inherited, introgression provides the most logical and effective approach to gene transfer, assuming that interspecific or intergeneric hybridization can be achieved. The probability of successfully hybridizing different crop species increases when the species are more closely related.

*Sorghum* is considered one of the closest relatives of the *Saccharum* complex, having diverged from a common ancestor as little as five million years ago (Al-Janabi et al., 1994). Guimaraes et al. (1997) illustrated this relationship by showing colinearity of 190 RFLP probes on genetic maps of *Sorghum* and *S. officinarum*. This close relationship has

been recognized for some time as *Saccharum* x *Sorghum* crosses have been reported with limited success (Venkatraman and Thomas, 1932; Bourne, 1935; Moriya, 1940; De Wet et al., 1976; Nair, 1999). Bourne (1935) attempted *Sorghum x Saccharum* crosses, (with sorghum as the female parent) but was not successful. More recently, Nair (1999) reported on the production of progeny from a *Sorghum* x *Saccharum* hybridization, but the frequency of viable progeny was low. From 3,670 well-pollinated florets only five seedlings were recovered. While there is obvious interest in creating and utilizing these hybrids between the two species, progress could be hastened by increased seed set and the ability to make selections among the resulting progeny.

The primary barrier to interspecific and intergeneric hybridization in sorghum is prezygotic; pollen tubes of alien species cease growth in pistils of sorghum before reaching the egg (Hodnett et al., 2005). Laurie and Bennett (1989) identified a sorghum trait, *iap* (*inhibition of alien pollen*), that permitted maize pollen tube growth to continue through the ovary to the micropyle when the sorghum female was homozygous for *iap*, but the recovery of sorghum-maize hybrids was not reported. Price et al. (2006) discovered that the same *iap* mutant removes the reproductive isolation between sorghum and several closely related wild taxa (*S. angustum*, *S. macrospermum* and *S. nitidum)* allowing the relatively easy production of new interspecific hybrids. Following this work, Kuhlman et al. (2008) documented the backcrossing of the previously described *S. macrospermum* hybrid to cultivated sorghum through the derivation of stable inbred lines with confirmed introgression from *S. macrospermum*. This introgression proves that large segments of chromosomes can be moved across Poaceae species, which can facilitate the intergeneric transfer of important and quantitatively inherited traits.

Given the potential benefits to sugarcane and sorghum crops and the renewed interest in both crops as bioenergy feedstocks, there is a logical interest in hybridization to combine their desirable characteristics. These characteristics include, but are not limited to, drought tolerance and wide adaptation from sorghum along with sugar concentration and perennial growth habit from sugarcane. Another potential benefit of wide hybridization between the species is the possibility of introducing the seed production capacity of sorghum into sugarcane and, in the long-term, developing a sugarcane variety that can be planted from true botanical seed as opposed to the current labor-intensive whole-stalk or billet planting methods. The objective of this study was to determine if sorghum germplasm possessing the *iap* mutant can be used to increase the frequency of sorghum/sugarcane hybrids and to assess the relative effect of sugarcane pollinators on seed set and progeny viability.

**MATERIALS AND METHODS**

*Production of Sorghum/Sugarcane Hybrids*: Seed of Tx3361, a line homozygous for *iap* and segregating for male sterility (Kuhlman and Rooney, in review), was planted in pots in the greenhouse from mid-July through mid-September to ensure flower synchronization between the sorghum and sugarcane plants. At the onset of anthesis, male sterile plants of Tx3361 were identified based on anther phenotype and isolated from unknown pollen by covering with a paper bag. Sorghum/sugarcane pollinations were made at the USDA-ARS Sugarcane Research Unit in Houma, Louisiana between late September and early November of 2007 and 2008. Additional pollinations were made in College Station, Tx in January and February of 2009. Tx3361 was used as the

female parent.  A total of 67 basic and commercial sugarcane breeding clones were used as pollen parents.

In 2007 pollinations made in Houma were completed by dusting the sorghum panicle with freshly collected sugarcane pollen and by rubbing the sorghum panicle through the sugarcane tassel.  Male parents included one commercial sugarcane cultivar, one released energy-cane cultivar (high-fiber sugarcane for biofuel production), three commercial breeding clones, four *S. spontaneum* accessions, and one *Erianthus* accession.  Also included were six breeding clones that resulted from the following crosses: one *S. spontaneum* x sugarcane ($F_1$), one *S. officinarum* x sugarcane ($F_1$), one *S. spontaneum x S. spontaneum*, two $F_1$ x sugarcane ($BC_1$);, and one $BC_1$ x sugarcane ($BC_2$).  In addition, one cross was made using multiple male parents (a polycross).  In 2008, crosses were made by tapping tassels of a single sugarcane parent over the top of one to three sorghum panicles.  To improve pollen load on the panicle, this was followed by rubbing the sorghum panicles into the sugarcane tassels.  For a single cross, pollinations were repeated for 3-4 consecutive days during sugarcane anthesis.  Males included five commercially released sugarcane cultivars, 24 sugarcane breeding clones, two *Erianthus* accessions, one *S. spontaneum* accession, and 13 basic breeding clones. The basic breeding lines included 12 $F_1$ hybrids between *S. spontaneum* and sugarcane and one $BC_2$. One polycross was also included in 2008.  Pollinated sorghum plants were returned to College Station for seed development and maturation.  The sorghum x sugarcane crosses made in College Station were completed using five commercial sugarcane breeding clones from the Texas AgriLife sugarcane breeding program in Weslaco, TX.

Each sorghum panicle was pollinated only one time using the techniques developed in Houma in 2008.

*Seed Harvest and Germination*: Seed was allowed to develop and mature for 46, 41, and 27 days post pollination in 2007, 2008, and 2009, respectively.  Seed from 2007 was stored from 30 to 90 d prior to germination.  A high frequency of vivipary was observed in 2007 resulting in a loss of hybrids.  To eliminate this problem in 2008 and 2009, seeds were not stored but were immediately germinated.  Prior to germination seeds were surface sterilized by soaking them in a liquid suspension of Captan$^{TM}$ and Apron$^{TM}$ (Syngenta, Wilmington, DE) for at least half an hour and then immersing them in a 30% solution of Chlorox$^{TM}$ (Proctor and Gamble, Oakland, CA) bleach for 20 minutes. Following surface sterilization, seeds were rinsed in sterile water and placed embryo side up in a petri dish containing a culture medium of Murashige-Skoog basal salts and vitamins (Murashige and Skoog, 1962) supplemented with 10 mg L$^{-1}$ glycine, 10 mg L$^{-1}$ L-arginine-HCl, 10 mg L$^{-1}$ L-tyrosine, 100 mg L$^{-1}$ inositol, and 30 g L$^{-1}$ sucrose, solidified with 0.7% agar (plant tissue culture grade, Phytotechnology Laboratories, Shawnee Mission, KS) (Sharma, 1999).  Petri dishes were maintained between 27 and 30 C under Gro-Lux$^{TM}$ flourescent lights (Sylvania, Danvers, MA) set to 14 h d$^{-1}$.  All seeds that showed good root and shoot development were placed in 10.2 cm pots.  Once established, plants were transferred to the greenhouse.

*Confirmation of Intergeneric Hybrid Plants*: Intergeneric hybrids were initially classified by morphology.   As they developed, all hybrids exhibited numerous characteristics of sugarcane (e.g. height, tillering, and maturity) that the maternal parent did not possess.  Plants assumed to be hybrids based on morphology were confirmed

using somatic chromosome numbers.  Chromosome spreads were prepared from root tips using a method described by Jewell and Islam-Faridi (1994) with the following modifications. Young actively growing root tips were pretreated with a saturated aqueous solution of α-bromonaphthalene for 2.75 h at room temperature and fixed overnight in 95% ethanol/glacial acetic acid (3:1 v/v).  Following fixation, root tips were rinsed several times with distilled water, hydrolyzed for 10 min in 0.2 M HCl and again rinsed in distilled water for 10 min.  Cell walls were digested for 35 to 60 minutes at 37 C with an aqueous solution of 5% cellulase (Onozuka R-10, Yakult Honsha Co. Ltd., Tokyo) and 1.0% pectolyase Y-23 (Seishin Corporation, Tokyo) at pH 4.5 and subsequently rinsed three times with distilled water.  Meristems were placed on a clean glass slide in an ethanol/glacial acetic acid (3:1) solution, macerated and spread with fine-tipped forceps, air-dried at room temperature for 2 d, and stained with Azure Blue.  Root tip spreads were examined using a Zeiss Universal II microscope (Carl Zeiss Inc., Gottingen, Germany) with 63X and 100X apochromat objectives.  Images were captured with an Optronics VI-470 system (Optronics Inc., Goleta, CA) and digitally stored and processed with Optimas (v. 6.1) image analysis software (Optimas Corp., Bothell, WA).

*Effect of Sugarcane Pollinator on Hybrid Seed Set:* For each cross made in Houma in 2008, the sugarcane parent, date of pollination, location of pollination, pollen rating, florets/panicle, seeds/panicle and seedlings produced were recorded.  Pollen rating was a subjective measurement determined at the time hybrid seed was harvested by observing the amount of pollen present on stigmas of the sorghum panicle.  The amount of pollen present on the stigmas was observed under a dissecting microscope and scored as 1, 2, or

3 with 1 being the least and 3 being the most.  For each cross made in College Station in 2009 the sugarcane parent, seeds/panicle and seedlings produced were recorded.

To determine relative effect of location, date of pollination and sugarcane pollinator on seed set and pollen rating, PROC GLM in SAS v9.1 was used.  Only sugarcane males that had been used in at least three pollinations were included in the analysis.  All effects were considered fixed and only interactions involving the pollinator were included the analysis of variance.

## RESULTS

*2007 Hybrid Seed Production, Confirmation and Growth*: In the fall of 2007, a total of 24 pollinations were made using 17 different pollinators (Table 1).  Based on stigma reaction, it was apparent by two to three days post pollination that fertilization had occurred.  Seed development was slower and the size was smaller when compared to intraspecific hybridization of sorghum.  Embryo loss during seed development, and vivipary after development, became evident when the seed was prepared for germination. Further analysis revealed that these were common problems with 39% of the seed having no embryo, and 32% being viviparous.  Seedlings were confirmed as intergeneric hybrids through chromosome counts.  As the seedlings progressed in development, it became evident that they represented a wide range of phenotypes, which ranged from very poor in growth to highly vigorous.

From these pollinations, 23 hybrids were transplanted to pots and placed in the greenhouse.  Somatic chromosome counts for these hybrids ranged from 56 to 64 (Fig. 1C). These hybrids displayed a wide range of phenotypes that included traits from both

*Saccharum* and Sorghum. All had numerous long narrow leaves like sugarcane and most tillered profusely. Two hybrids, L07-9S (Tx3361 x HoCP04-838) and L07-11S (Tx3361 x US06-9025) showed more vigorous growth than the others. In seven months, stalks of hybrid L07-9S were 2.7 m in height and those of L07-11S were 3.1 m (Fig. 1A) compared to the mean height of 1.1 m for the maternal Tx3361. Unlike Tx3361, both hybrids were photoperiod sensitive like sugarcane, and flowered from mid December through January in College Station whereas Tx3361 flowers in approximately 65 d regardless of planting date. The panicles on L07-9S and L07-11S were slightly more compact than those of sugarcane (Fig. 1B), and appeared male sterile; attempted pollinations onto Tx3361 did not produce seed. In August, several stalks of each hybrid were cut to test for the ability to vegetatively propagate and to assess the accumulation of soluble sugars and their distribution. Vegetative propagation through nodal cuttings was successful and internode brix values ranged from 8.5 to 19% with concentrations increasing with internode maturity as is seen in sugarcane (Whittaker and Botha, 1997).

*2008/2009 Hybrid Seed Production and Enhancement of Process*: In 2008 a total of 155 sorghum panicles (totaling 74,300 florets) were pollinated. From these pollinations, 10,347 seed were recovered, resulting in an average seed set of 14%. Percent seed set was not measured in the 2009 pollinations, but it appeared similar to that observed in 2008. Seed was harvested 40 d and 28 d post pollination in 2008 and 2009, respectively. Germination rates for the 2008 seed still suffered some from vivipary. In addition it was discovered that many of the embryos could not grow through the seed coat, which further limited germination rates in this year. In 2009 an additional decrease in maturation time further reduced vivipary, and excising the pericarp prior to planting removed the seed

coat barrier. These minor modifications significantly improved germination rates from 2.5% in 2007, to 5.7% in 2008, and to a much improved 33% in 2009.

From the combined 2008/2009 pollinations, a total of 1348 seedlings were transplanted to the greenhouse. The phenotypic variation present in these hybrids was extensive, but all were morphologically more like sugarcane than sorghum. These hybrids are expected to follow growth and development patterns observed in the limited set of hybrids evaluated from the 2007 crosses.

*Effect of Pollinator Parent on Seed Set and Germination*: Analysis of variance detected a significant effect of pollinator parent on seed set (Table 2), indicating that the source of sugarcane pollen is critical in the success of the production of intergeneric hybrids with Tx3361. Tx3361 had good seed set when pollinated with sugarcane clones L06-024, HoCP05-904, Ho06-562 and L01-283 which had seed set rates of 53.0%, 36.0%, 25.2%, and 24.9%, respectively. These pollinators are of particular interest for the production of intergeneric hybrids, while other clones with poor seed set percentages (i.e. <10%) should be avoided (Table 3).

Pollen rating in sugarcane (Table 2) is influenced by genotype and environment, with the date of pollination having a significant effect on pollen shed (Moore and Nuss, 1987). In our study, clones with a low mean pollen rating consistently produced crosses with low seed set, but using clones with a high mean pollen rating did not necessarily produce high seed set. Six of the top seven sugarcane pollinators (defined by seed set percentage) had an average or above average mean pollen rating, while mean pollen ratings in males producing below average seed set varied (Table 3). These results imply

that males must not only produce high pollen ratings but that they must also have favorable genetic and/or genomic compatibility with Tx3361.

Analysis of variance of the 2009 data indicated that once the seed was set, neither pollination environment nor sugarcane pollinator influenced percent germination. Based on the current methods of managing seed production and germination, it is reasonable to expect between 25-40% of seed to be viable regardless of which pollinator is used and where the pollination is made.


## DISCUSSION

An average seed set of 53% when using sugarcane pollinator L06-024 was unexpectedly high for an intergeneric cross, considering attempts by previous researchers resulted in no more than a few plants (Nair, 1999). The high rate of seed production is attributed to the elimination of pre-fertilization barriers through the use of Tx3361 as well as compatibility of this line with particular sugarcane pollinators. Once produced, management of the hybrid seed prior to germination was critical to maximize production. Marked increases in viable seedlings were observed in each successive crossing year as problems affecting germination were identified. These increases resulted from the elimination of vivipary and physical barriers through early harvest and the removal of the pericarp.

Eliminating hybridization barriers and improving the germination rate has substantially increased the capacity to generate hybrids when compared to previous work. Nair (1999) "thoroughly pollinated" 3,670 florets and produced five seedlings for a success rate of 0.13%. In 2008, 16,813 florets were pollinated using males with a high

pollen rating. Of these pollinations, 162 plants were produced for a success rate of 1%. Assuming that "thoroughly pollinated" is equivalent to a high pollen rating, this represents a 7.7-fold increase in plant recovery between the 2008 crossing season over results reported by Nair (1999). As modifications were made to the seed treatment, an additional 6-fold increase in recoverable progeny was achieved in 2009. Thus, the combined increases resulted in approximately a 40-fold increase in recovered progeny when compared to the previous report.

A limited number of male parents were screened in the current study. It is logical to assume that further screening will uncover additional compatible sugarcane pollinators that will expand production of intergeneric hybrids by increasing seed set and by improving seed quality. Therefore continued screening of *Saccharum* pollinators will be necessary to identify the best males for intergeneric hybrid production. The capacity to produce large-scale quantities of intergeneric *Sorghum*/*Saccharum* hybrids opens a wide range of possibilities for genetic improvement of sugar and bioenergy crops. While successful hybridization between sorghum and sugarcane, *S. spontaneum,* and early generation *Saccharum* hybrids, are described in this study, there is a need to determine the range of germplasm that can be hybridized using the developed lines and techniques. It may be possible to hybridize sorghum with other grasses of the Poaceae (e.g. *Miscanthus*, *Erianthus*, etc.) to facilitate introgression of positive traits among the genera/species.

The genetic and phenotypic variation present among the newly developed *Sorghum*/*Saccharum* hybrids presents significant opportunities. Given the amount of variation present and the large numbers of hybrids produced, segregation is expected to

allow for the selection of elite hybrids. Even in 2007, the lowest of the three reported years for seedling production, there was enough variation among the 23 viable seedlings to select two that were visually superior to the others based on agronomic type. Further characterization of these two selected seedlings, as well as characterization of future selections is necessary to determine unique strengths and weaknesses of the hybrids.

Selected hybrids can be used to introgress large genomic regions that control valuable quantitative traits from one species to the other. For example, the potential to transfer drought tolerance from sorghum to sugarcane or to introgress enhanced sugar production from sugarcane into sorghum could significantly influence energy and sugar production throughout the world. Because the initial $F_1$ hybrids did not produce seed when crossed with sorghum, cytological manipulations will likely be needed, but established procedures provide approaches to mitigate this obstacle (Kuhlman et al., 2008).

If the $F_1$ hybrids possess unique and desirable agronomic characteristics and they perform well in agronomic trials, there is the potential to develop a new intergeneric hybrid crop. For example, a "sorcane" hybrid with high sugar accumulating capacity and enhanced water-use efficiency may be valuable as either a seed or vegetatively propagated crop. Additional research and development on sorghum seed parents and sugarcane pollinators must be completed to maximize seed production and development to make seed propagation a viable option. However, the germplasm and techniques described will produce seed quantities suitable for introgression, selection, and testing purposes.

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Table 1.  Sugarcane parents used in the sorghum x sugarcane crosses listed by year and location of cross.  Number of panicles pollinated, number of seed produced and number of seedlings grown are listed by pollinator.  Male parents are described by generation as released energy cane (REC), commercial breeding clone (CBL), released sugarcane (RSC), *S. spontaneum* (spontaneum), *Erianthus,* $F_1$, $BC_1$, $BC_2$, or polycross.  Total florets